REMARKS

Claims 20-21 and 24-27 are in this application. Claims 25-27 are free of the prior art.

The Examiner alleges that there is no written description of Claims 20-21 and that these claims are not enabled. This is respectfully traversed.

Claim 20 defines a bioemulsifier from any *Acinetobacter* strain isolated from human skin that retains 35% stability after 140 hrs at 10°C. This claim is specific to *Acinetobacter* strains isolated from human skin. The properties of *Acinetobacter* from human skin are unique to the microenvironment of human skin. The attached article Seifert et al., "Distribution of Acinetobacter Species on Human Skin: Comparison of Phenotypic and Genotypic Identification Methods.", Journal of Clinical Microbiology, Nov. 1997, p. 28109-2825 supports applicants' position that there is a written description to support Claims 20-21 and that Claims 20-21 are enabled. This paper is also cited in the specification of this application. This paper discloses that not all strains of *Acinetobacter* are isolated from the skin so that a bioemulsifier isolated from an *Acinetobacter* strain that can be isolated from human skin is clearly described and enabled.

Therefore, it is respectfully requested that the rejection under 35 USC 112, first paragraph be withdrawn.

The Examiner's statement that Claims 20-21 and 24 are indefinite under 35 USC 112, second paragraph, is traversed.

It is applicants' position that there seems to be some confusion regarding the term emulsion

stability. The bioemulsifier is able to emulsify the oil. The oil once emulsified remains stable for a long period of time i.e. 140 hours. This is an excellent result. That is to say 35% emulsification activity is still retained even after standing for 140 hours. In addition, the emulsion maintains 90% of its stability upto 6 days at 37°C, in addition to the stability already mentioned at 10°C. The concept of emulsion stability is discussed in applicants' paper Patil, J.R. and B.A. Chopade (2001) Journal of Applied Microbiology 2001, 91, 290-298, see page 293.

As to Claim 24, an esterase is an enzyme that is involved in the release of bioemulsifier from a cell. This enzyme is not part of a protein component of the bioemulsifier.

The esterase activity was checked in the free cell supernatant as well as in the cell bound form. Esterase activity was associated with both forms.

The kinetics and distribution of esterase produced by SC14 was studied. This suggests the involvement of esterase in the release of emulsifying factor from the cell surface into the fermentation medium.

Therefore, it is respectfully requested that this rejection be withdrawn.

According to the Examiner Claim 20 is rejected as being anticipated or in the alternative, as being obvious in view of Gutnick (US Patent 4,230,801). This is respectfully traversed.

The natural habitat of the *Acinetobacter* ssp discussed in Gutnick is different than the human skin. As disclosed in column 4, lines 21-59 of the patent, the extracellular microbial

lipopolysaccharide produced was a function of the medium used to grow the *Acinetobacter* ssp and none of these are human skin.

According to Gutnick, the *Acinetobacter* bioemulsifier produced by growing *Acinetobacter* Sp. ATCC 31012 (also known as strain RAG-1) on crude oil or hexadecane is an extracellular microbial protein-associated lipopolysaccharide that was identified as ".beta.-emulsan" and given the common name "protoemulsans". The lipopolysaccharide is an – and O-lipoacylated heteropolysaccharide made up of amounts of D-galactosamine and an aminouronic acid, the O-lipoacyl portion of the lipoheteropolysaccharide containing from 2 to 3 percent by weight of various fatty acid esters in which (a) the fatty acids contain from about 10 to about 18 carbon atoms; and (b) less than 50 percent by weight of such fatty acids are composed of 2-hydroxydodecanoic acid and 3-hydroxydodecanoic acid. The growth of *Acinetobacter* Sp. ATCC 31012 on ethanol as the primary assimilable carbon source yields a significantly different extracellular microbial protein-associated lipopolysaccharide, which Gutnick identified as ".alpha.-emulsans", in which the lipopolysaccharide is also an – and O-lipoacylated heteropolysaccharide made up of major amounts of D-galactosamine and an aminouronic acid, but in which the O-lipoacyl portion of the lipoheteropolysaccharide contains at least 5 percent by weight (and, more often, between 7 to 14 percent by weight and occasionally as high as 19 percent by weight) of various fatty acid esters in which (a) the fatty acids contain from about 10 to about 18 carbon atoms which are usually distributed in different ratios than those in the low-ester protoemulsans; and (b) more than 50 percent by weight or more of such fatty acids are composed of 2-hydroxydodecanoic acid and 3-hydroxydodecanoic acid. The .alpha.-emulsans rather than .beta.-emulsans may also be produced by growing *Acinetobacter* Sp. ATCC 31012 on fermentation media containing one or more fatty acid salts as the primary assimilable carbon source. In addition, according to Gutnick, the .alpha.-emulsans are much more effective than .beta.-emulsans

in the emulsification of various crude oils and gas-oils and, in some instances (such as the emulsification of Bunker C fuel oil), efficiently form stable emulsions where .beta.-emulsans have no effect.

Furthermore, it is noted that bioemulfier of Gutnick is checked over a range from 20 to 35°C while the bioemulsifier claimed in Claim 20 is produced at a temperature of 37°C.

It is noted that all bioemulsifiers have the properties of reducing viscosity so the disclosure in Gutnick of reducing viscosity is certainly not sufficient for the disclosure to anticipate or make obvious Claim 20.

Therefore it is respectfully requested that this rejection be withdrawn.

The Examiner alleges that Claims 20 and 21 are obvious in view of Gutnick (US patent 4,230,801); Shabtai et al, Zosim et al and Pola Kasei Kogyo. This is respectfully traversed.

As stated on page 1 of the specification *Acinetobacter* strains from human skin exhibited better emulsification activity than those obtained from a burn wound or soil isolates. The fact that only specific strains of *Acinetobacter* are found on the skin surface is supported by the Seifert et al. article referred to above. As set out in the specification, *Acinetobacter RAG-1* has not been isolated from the skin so Zosim and Shabtai cannot be combined with Gutnick and the other references cited by the examiner. The combination of these references with Pola Kasei Kogyo does not make the invention of Claims 20 and 21 obvious.

Therefore, it is respectfully requested that this rejection be withdrawn.

Accordingly, it is submitted that the application is in condition for allowance and favorable consideration is respectfully requested.

Respectfully submitted,

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Distribution of *Acinetobacter* Species on Human Skin: Comparison of Phenotypic and Genotypic Identification Methods

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At least 19 genomic species are recognized as constituting the genus *Acinetobacter*. However, little is known about the natural reservoirs of the various members of the genus. An epidemiological study was therefore performed to investigate the colonization with *Acinetobacter* spp. of the skin and mucous membranes of 40 patients hospitalized in a cardiology ward and 40 healthy controls. Single samples were obtained once from each of nine different body sites, i.e., forehead, ear, nose, throat, axilla, hand, groin, perineum, and toe web. Identification of *Acinetobacter* isolates was achieved by using phenotypic properties and was compared to identification by amplified ribosomal DNA restriction analysis. Selected isolates were further investigated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ribotyping, and DNA-DNA hybridization. Plasmid profile analysis was used for epidemiological typing. Thirty patients (75%) and 17 controls (42.5%) were found to be colonized with *Acinetobacter* spp., and the colonization rates of patients increased during their hospital stay. The most frequently isolated species were *Acinetobacter lwoffii* (47%), *A. johnsonii* (21%), *A. radioresistens* (12%), and DNA group 3 (11%). In contrast, *A. baumannii* and DNA group 13TU, the most important nosocomial *Acinetobacter* spp., were found only rarely on human skin (0.5 and 1%, respectively) and their natural habitat remains to be defined. A good correlation between phenotypic and genotypic methods for identification of *Acinetobacter* spp. was observed, and only two isolates could not be assigned to any of the known DNA groups.

Numerous reports have illustrated the increasing nosocomial problem posed by *Acinetobacter* species. These organisms are generally regarded as ubiquitous microorganisms, since they are found frequently in soil, water, and dry environments and have also been isolated from the hospital environment, foods, and animals. In addition, *Acinetobacter* species are apparently the only group of gram-negative bacteria that may be natural residents of human skin (16, 19, 20).

It was not until the taxonomy of the genus had been revised by Bouvet and Grimont (6, 7) that a detailed investigation of the epidemiology of *Acinetobacter* spp. became possible. At least 19 genomic species have been recognized, 11 of which can be differentiated by phenotypic properties. The majority of the studies have, however, concentrated on the hospital epidemiology of these organisms. It has been shown that most clinical isolates belong to *Acinetobacter baumannii* and that this species is involved in the majority of nosocomial infections and hospital outbreaks (4, 5, 28). However, little is known about the natural reservoirs of the various *Acinetobacter* species.

To further assess the natural habitats of the different members of the genus, we investigated the colonization with *Acinetobacter* species of the skin of hospitalized patients and healthy volunteers.

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MATERIALS AND METHODS

Study population. We studied two groups of people, i.e., 40 patients hospitalized in a 20-bed regular ward of the cardiology department of a large university hospital in Cologne, Germany, and 40 healthy controls, 9 of whom were laboratory workers. Patients had a mean age of 61 years (range, 33 to 85 years) and were hospitalized for various diseases, such as coronary heart disease, rheumatic heart disease, or arrhythmias, or had undergone invasive procedures such as percutaneous transluminal coronary angioplasty and coronary bypass surgery. Healthy volunteers had a mean age of 31 years (range, 23 to 55 years). Thirty of the patients were male, whereas 14 of the controls were male.

Bacterial isolates. Patients and controls were sampled once during two 3-month study periods, from 1 July to 30 September 1993 and from 1 January to 31 March 1994. Cotton swabs premoistened with sterile saline were rubbed vigorously, with rotation, over approximately 6-cm^2 sites. Swabbing was performed on multiple body sites including forehead, ear (external auditory canal), nose (anterior nares), throat, axilla, hand, groin, perineum, and toe web. A total of 720 samples were collected. Swabs taken were streaked onto blood agar plates and cultured semiquantitatively at 30°C for 48 h. Blood agar plates supplemented with vancomycin (4 mg/liter) were inoculated in parallel to suppress the gram-positive skin flora. Swabs were also inoculated into 40 ml of a liquid enrichment medium containing sodium acetate as the sole carbon source, as described by Baumann (3). Enrichment cultures were incubated in 250-ml Erlenmeyer flasks at 30°C on a rotary shaker (200 rpm) for 48 h with vigorous aeration and were subsequently plated onto solid media as described above. Isolates were presumptively identified as *Acinetobacter* species by Gram's stain, motility testing, a negative oxidase reaction, and the oxidation-fermentation test. Identification of isolates at the genus level was confirmed by the transformation assay of Juni (18). Phenotypic identification was performed by using the simplified identification scheme of Bouvet and Grimont (6) including growth in tryptic soy broth at 37, 41, and 44°C; production of acid from glucose in oxidation-fermentation test medium; gelatin hydrolysis; and assimilation of 14 different carbon sources.

Plasmid isolation. Strain identity among organisms isolated from different body sites of a given patient was confirmed by plasmid profile analysis. Plasmid DNA was prepared by the method of Hartstein et al. (17), with minor modifications as described previously (24). In brief, cells from an overnight culture were suspended in 3 ml of 2.5 M NaCl–10 mM EDTA (pH 8.0). Cell walls were weakened with lysozyme (10 mg/ml). A lysis solution containing 0.5 ml of 0.5% mixed alkyltrimethylammonium bromide and 0.5 ml of 1% Triton X-100 was

added. The resulting lysate was incubated in a water bath at 56°C. Following protein extraction and alcohol precipitation of plasmid DNA, samples were electrophoresed in 0.7% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.2]) for 18 h at 30 V. Gels were stained with ethidium bromide and photographed under a UV lamp. A plasmid type was defined as any plasmid pattern which varied from another pattern with regard to the number and size of plasmid bands. Isolates were considered similar if one of the compared patterns contained one or two additional bands. Isolates were run in duplicate on different gels. For comparison and reproducibility testing, one strain was retested on each gel.

ARDRA. The amplified ribosomal DNA restriction analysis (ARDRA) method was carried out as described previously (33). Briefly, strains were grown on Mueller-Hinton Agar II (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. A 1-μl loopful of colony growth was suspended in 20 μl of 0.05 M NaOH–0.25% sodium dodecyl sulfate (SDS) solution and heated at 95°C for 15 min, diluted to a 200-μl total volume, agitated thoroughly, and centrifuged briefly in a benchtop microcentrifuge at 13,000 × *g*. The supernatant was used as the target DNA in a PCR. The sequences of the primers were 5'TGGCTCAGATT GAACGCTGGCGGC (5' end of the 16S rRNA gene) and 5'TACCTTGTTA CGACTTCACCCCA (3' end of the 16S rRNA gene). Amplification, restriction digestion, and electrophoresis were carried out as described previously (33). The restriction enzymes *Alu*I (AGCT) and *Cfo*I (GCGC) were used for all isolates. Depending on the outcome of these tests, further identification was carried out by restriction with *Mbo*I (GATC), *Rsa*I (GTAC), *Msp*I (CCGG), *Bfa*I (CTAG), and/or *Bsma*I (GTCTC). Restriction fragment patterns were analyzed by gel electrophoresis of 8 μl of each restriction mixture at 7 V/cm for 3 h in 3% (wt/vol) Metaphor agarose (FMC BioProducts, Rockland, Maine) in Tris (89 mM) boric acid (89 mM) EDTA (2 mM) electrophoresis buffer, pH 8.0. Gels contained 50 ng of ethidium bromide/ml. Gels were photographed, and patterns were compared visually.

DNA-DNA hybridization. DNA-DNA hybridization was performed by using a filter dot method as described previously (31). The same reference strains of the known *Acinetobacter* DNA groups were used as in previous studies, and a strain was identified as belonging to a certain DNA group on the basis of previously published criteria (15, 31, 32).

Cell envelope protein electrophoretic typing. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cell envelope proteins was performed as described earlier (8, 9). Briefly, cell envelope fractions were obtained by ultrasonic disruption of cells and subsequent fractional centrifugation. SDS-PAGE was performed by using a 3% stacking gel and an 11% acrylamide running gel. Fast Green FCF (Sigma Chemical Co., St. Louis, Mo.) was used for staining, and patterns were compared visually to patterns of a set of 120 strains (9) belonging to DNA groups 1 to 12 (7) and groups 13TU and 13BJ/14TU (32).

Ribotyping. Ribotyping was performed with the restriction enzyme *Eco*RI as described previously (12). In brief, cells were lysed by a modified EDTA-SDS method. DNA was extracted with phenol-chloroform and digested with *Eco*RI or *Cla*I. After agarose gel electrophoresis, the separated fragments were transferred to a nylon membrane and hybridized with a digoxigenin-11-dUTP-labelled cDNA probe derived from a commercially available *Escherichia coli* 16S and 23S rRNA preparation by random priming using reverse transcriptase. The following reference strains were also ribotyped: NCTC 5866^T, ATCC 17968, 284, and 44 (*A. lwoffii*); 151a and 118 (DNA group 15TU); ATCC 17909^T (*A. johnsonii*); and ATCC 17922 (DNA group 3) (15).

RESULTS

Isolation rates and biochemical species identification. A total of 186 *Acinetobacter* isolates were recovered from 30 patients (155 isolates) and 17 controls (31 isolates), giving colonization rates of 75% for patients and 42.5% for controls. The colonization rates of male and female patients (73.3 and 80%) and those of male and female controls (42.3 and 42.9%) were similar. No seasonal variation was observed. Remarkably, growth of the majority of organisms on primary isolation was only slight (50%) or moderate (10%) and 40% of the isolates grew only after enrichment. The number of CFU on blood agar was usually 1 to 2 $\log_2$ lower than for concomitant skin organisms such as coagulase-negative staphylococci and coryneforms. Table 1 shows the species distribution as determined by phenotypic properties for both patients and controls. The most frequently isolated species were *A. lwoffii* (*n* = 87; 47%), *A. johnsonii* (*n* = 40; 21%), *A. radioresistens* (*n* = 22; 12%), and *Acinetobacter* genomic species 3 (*n* = 20; 11%), whereas *A. junii* (*n* = 9; 5%), *A. baumannii* (*n* = 3; 1.5%), and *Acinetobacter* genomic species 10 (*n* = 1; 0.5%) were detected only rarely. Only four isolates (2%) could not be identified by phenotypic properties, giving an identification rate of 98%. *A. calcoaceticus*, *A. haemolyticus*, and unnamed *Acinetobacter* genomic species 6 and 11 were not found on human skin or mucous membranes.

TABLE 1. Distribution of different *Acinetobacter* spp. identified by phenotypic properties after isolation from the skin and mucous membranes of patients and controls

Genomic species (DNA group)[a]	No. (%) of *Acinetobacter* strains isolated from:		
	Patients	Controls	Both groups
A. baumannii (2)[b]	2 (1)	1 (3)	3 (1.5)
Acinetobacter sp. 3	18 (12)	2 (6)	20 (11)
A. junii (5)	6 (4)	3 (10)	9 (5)
A. johnsonii (7)	34 (22)	6 (20)	40 (21)
A. lwoffii (8/9)[c]	69 (44)	18 (58)	87 (47)
Acinetobacter sp. 10	1 (1)	0 (0)	1 (0.5)
A. radioresistens (12)	22 (14)	0 (0)	22 (12)
Unidentified	3 (2)	1 (3)	4 (2)
Total no. of isolates	155	31	186

[a] According to references 7 and 32.
[b] Two of these isolates were later identified as belonging to DNA group 13TU.
[c] One of these isolates could later not be assigned to any of DNA groups 10 to 15TU.

Interestingly, the number of colonized body sites was greater in patients than in controls (2.8 colonized sites per patient versus 0.7 sites per control). Twenty patients and six controls were colonized with two or more different *Acinetobacter* spp. at different body sites, with a maximum of eight (of nine) body sites colonized with *Acinetobacter* spp. and a maximum of five different species recovered from one patient. A given body site was colonized with two or more different *Acinetobacter* spp. in 12 patients and two controls. The most frequently colonized body sites were the hands (26%), the groin (25%), toe webs (24%), the forehead (23%), and the ears (21%) (Table 2).

Plasmid profiles. Strains of a given species recovered from unrelated controls were thought to be epidemiologically unrelated, and typing of these was not considered to be necessary. Therefore, only isolates obtained from hospitalized patients (*n* = 155) were further investigated by plasmid analysis. Plasmids were found in all but three isolates (98%), and 104 unique profiles were obtained. There were 2 plasmid types in 2 *A. baumannii* isolates, 9 plasmid types among 18 *Acinetobacter*

TABLE 2. Rates of colonization of various body sites of patients and controls with *Acinetobacter* spp.

Body site	No. (%) of patients or controls colonized with *Acinetobacter* spp.		
	Patients	Controls	Both groups
Forehead	13 (33)	5 (13)	18 (23)
Ear	14 (35)	3 (8)	17 (21)
Nose	13 (33)	3 (8)	16 (20)
Throat	6 (15)	0 (0)	6 (8)
Axilla	13 (33)	1 (3)	14 (18)
Hand	13 (33)	8 (20)	21 (26)
Groin	15 (38)	5 (13)	20 (25)
Perineum	8 (20)	1 (3)	9 (11)
Toe web	16 (40)	3 (8)	19 (24)
Total no. (%) of sites colonized	111 (31)	29 (8)	140 (19)

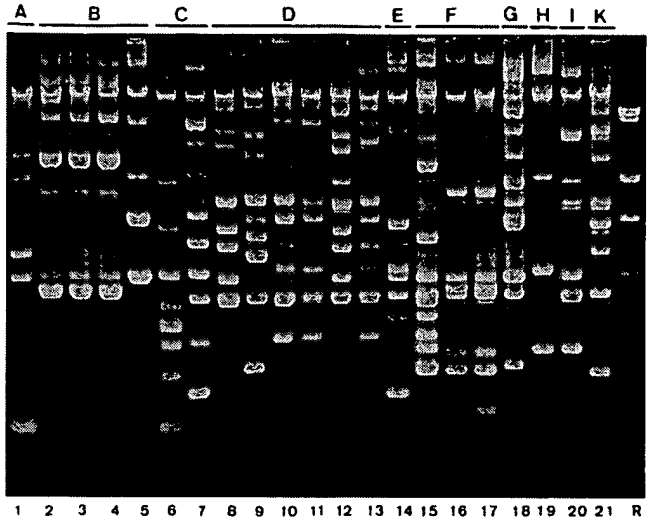

FIG. 1. Plasmid profiles of 21 *A. johnsonii* strains isolated from 10 patients (A to K). Identical or closely related profiles were obtained only in a given patient. Up to three different strain profiles of the same species were found in one patient (D). Lane R contained a molecular size marker (*Hin*dIII digest of lambda DNA).

TABLE 3. Comparison of phenotypic methods and ARDRA for identification of 138 *Acinetobacter* isolates recovered from skin and mucous membranes

Genomic species (DNA group[a]) based on biochemical identification	No. of isolates in DNA group (based on ARDRA):										
	2	3	5	7	"7"	8	10	12	13TU	15TU	NI
A. baumannii (2)	1								2		
Acinetobacter sp. 3		11									
A. junii (5)			8								
A. johnsonii (7)				10	17[b]						2[c], 2[d]
A. lwoffii (8/9)						60					1
Acinetobacter sp. 10							1				
A. radioresistens (12)								19			
Not identified		1				1				1	

[a] According to references 7 and 32.

[b] Isolates with a combination of *Cfo*I patterns 1 and 5 instead of the previously reported *Cfo*I 1 pattern. Presumptive identification by ARDRA as *A. johnsonii* based on the characteristic combination of restriction patterns obtained with the other enzymes used.

[c] Isolates with *Cfo*I 5 profile instead of *Cfo*I 1 profile. Although the combination of restriction patterns obtained with the other enzymes used was characteristic for *A. johnsonii*, no presumptive identification could be given.

[d] Isolates with aberrant *Bfa*I patterns such that no differentiation among DNA groups 4, 7, and 14TU and no final differentiation could be achieved.

genomic species 3 isolates, 5 plasmid types among 6 *A. junii* isolates, 25 plasmid types among 34 *A. johnsonii* isolates, 42 plasmid types among 69 *A. lwoffii* isolates, 17 plasmid types among 22 *A. radioresistens* isolates, 3 plasmid types among 3 unidentified *Acinetobacter* isolates, and 1 plasmid type in the single isolate identified as *Acinetobacter* genomic species 10.

Nineteen patients yielded the same *Acinetobacter* species, and often the same or a closely related strain, as determined by plasmid profile analysis, recovered from different sites. Sixteen patients harbored isolates of the same *Acinetobacter* species that were recovered from different body sites or even from the same body site—mainly *A. lwoffii* and *A. johnsonii*—but these exhibited up to four different plasmid profiles (Fig. 1). However, possible cross transmission of strains in the hospital setting, i.e., the isolation of at least two isolates with identical or closely related plasmid profiles in two patients, was documented only once.

ARDRA. A total of 138 isolates, comprising 107 isolates recovered from patients (including 104 isolates with unique plasmid profiles and the 3 isolates without plasmids) and 31 isolates from unrelated controls, were subsequently studied by ARDRA for confirmation of identification at the species level (Table 3).

Of the three *A. baumannii* isolates identified by phenotypic properties, two (SH 27 and SH 197) were identified by ARDRA as belonging to DNA group 13TU; biochemically, these two isolates were identified as *A. baumannii* biotypes 9 and 6, respectively. One isolate was identified as *A. baumannii* by both approaches and was characterized biochemically as biotype 6.

All 11 isolates that were identified phenotypically as belonging to DNA group 3 were also identified as DNA group 3 by ARDRA.

The eight isolates of *A. junii* were correctly identified by both methods. Previously, ARDRA did not differentiate between *A. junii* and DNA group 17TU but it has been shown recently that restriction with *Bfa*I allows differentiation between these two genomic species (10a).

The most problems were encountered with the use of ARDRA to differentiate *A. johnsonii* from *A. haemolyticus* and DNA group 14TU by restriction digestion with *Bfa*I (10a). Previously, no differentiation between these species by ARDRA was possible (33). However, in the present study, substantial intraspecific variability and some previously unobserved profiles were encountered for this species. Ten isolates were correctly identified as *A. johnsonii* by both methods. The *Cfo*I restriction profile observed for 17 isolates was a mixture of *Cfo*I profiles 1 and 5 instead of the previously reported *Cfo*I 1 profile (Fig. 2). These isolates were presumptively identified as "*A. johnsonii*" based on the characteristic combination of patterns obtained with other restriction enzymes (*Alu*I, *Mbo*I, *Rsa*I, and *Bfa*I). Furthermore, two isolates (SH 180 and SH 196) had a *Cfo*I 5 profile not observed previously in combination with the profile combination obtained with the other enzymes characteristic for *A. johnsonii*. These two isolates remained unidentified by ARDRA. Finally, two other isolates (SH 137 and SH 151) had an aberrant *Bfa*I pattern such that no final differentiation among *A. johnsonii*, *A. haemolyticus*, and DNA group 14TU could be made. Fifteen of these isolates with aberrant ARDRA profiles were tested by the other methods, and all were correctly identified as *A. johnsonii*. Regular and aberrant *Cfo*I ARDRA patterns are shown in Fig. 2, which also presents *Alu*I patterns 2 and 4, and the *Alu*I combination of patterns 2 and 4, which was found in a single strain (SH 213).

Sixty *A. lwoffii* isolates were correctly identified by both ARDRA and phenotypic tests. One isolate (SH 203) was identified phenotypically as *A. lwoffii* but remained unidentified by ARDRA and other methods. This isolate may belong to an as yet unidentified species, since it did not belong to any of DNA groups 1 to 15TU as determined by DNA-DNA hybridization (Table 4).

One isolate was identified phenotypically as belonging to DNA group 10, and this was confirmed by ARDRA. This method can now differentiate between DNA groups 10 and 11 on the basis of restriction of amplified 16S ribosomal DNA with *Bsma*I (10a).

All 19 *A. radioresistens* isolates were identified correctly by both biochemical tests and ARDRA.

Of the four isolates that could not be identified biochemi-

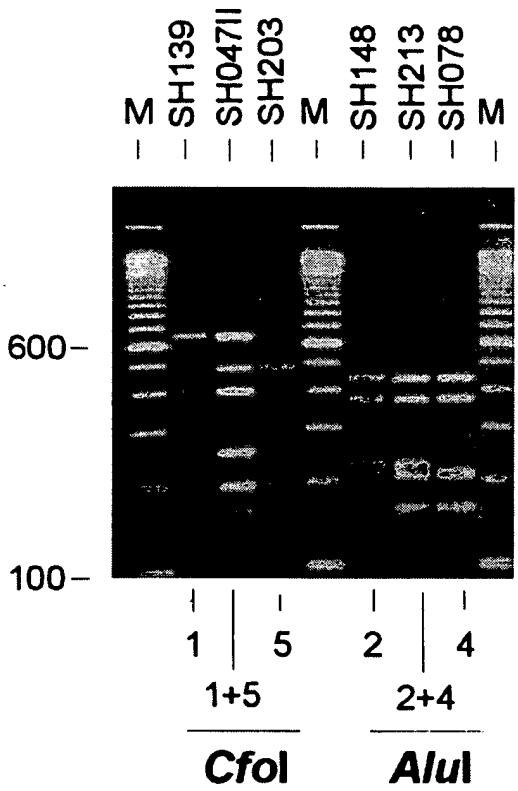

FIG. 2. ARDRA restriction patterns obtained with restriction enzymes *Cfo*I and *Alu*I. *Cfo*I 1, *Cfo*I 5, *Alu*I 2, and *Alu*I 4 are regular patterns; *Cfo*I 1+5 and *Alu*I 2+4 are aberrant patterns. For strain designations, see Table 4. Lane M, molecular size marker.

cally, one (SH 148) was identified as belonging to DNA group 15TU by ARDRA and another (SH 184) was identified as *A. lwoffii*. Both isolates were biochemically inactive. One isolate (SH 219) had an aberrant biochemical profile but was identified by ARDRA as belonging to DNA group 3. These identifications were confirmed by other methods. The remaining isolate (SH 139) could not be assigned to any of the described DNA groups by ARDRA, SDS-PAGE, and ribotyping. DNA-DNA hybridization showed that this isolate did not belong to any of DNA groups 1 to 15TU.

A total of 28 *Acinetobacter* isolates gave inconclusive results by biochemical and ARDRA identification because (i) they were not identified by at least one of the methods, (ii) both methods showed discrepant results, or (iii) isolates exhibited aberrant ARDRA patterns not observed before. Twenty-five of these isolates were further investigated with more laborious techniques such as DNA-DNA hybridization, which is the "gold standard" of bacterial species identification, and by SDS-PAGE and ribotyping, which have also been shown to enable appropriate identification (9, 14). For comparison, the identification results obtained by all five methods for these isolates are listed in Table 4.

DNA-DNA hybridization. DNA-DNA hybridization was performed with 24 isolates (strain SH 219 was not tested). Fifteen isolates were identified as belonging to DNA group 7 (*A. johnsonii*), six were in DNA group 8 (*A. lwoffii*), and one was in DNA group 15TU, while two isolates were not identified as belonging to any of DNA groups 1 to 15TU.

Identification by cell envelope protein electrophoresis. Cell envelope protein patterns were characterized by approximately 30 to 40 bands differing in staining intensity in the molecular weight range of 14,000 to 100,000. Strains with similar patterns were run repeatedly in adjoining lanes for final grouping with reference strains of known DNA groups included in these electrophoretic runs (9) (Fig. 3). For DNA group allocation, relatively weakly stained bands with molecular weights of >50,000 were used as the primary criterion (9), but the overall pattern was also taken into account. We identified 16 isolates as *A. johnsonii*, 5 as *A. lwoffii*, and one as either *A. johnsonii* or *A. lwoffii*, while two were not identified as belonging to any known DNA group.

Ribotyping. All of the reference strains of *A. lwoffii* displayed a small 1.1-kb band not seen in any other DNA group. All *A. johnsonii* strains showed at least two of three bands with sizes of 3.1, 2.5, and 1.4 kb. The DNA group 15TU strains showed two bands of 2.1 and 3.0 kb not displayed by other DNA groups. The DNA group 3 strain was identified by the presence of 0.8-, 2.4-, and 3.6-kb bands. Based on these characteristics, 23 of 25 isolates could be identified (Table 4). Two isolates (SH 139 and SH 203) were not identified.

DISCUSSION

Although it is largely accepted that microorganisms of the genus *Acinetobacter* are normal inhabitants of human skin (16, 19, 20), few studies have specifically addressed the colonization of human skin and mucous membranes with *Acinetobacter* spp. The skin was first studied as a potential reservoir of *Acinetobacter* spp. by Taplin et al. in 1963 (30). At that time, these organisms were known as *Mima polymorpha* and *Herellea vaginicola*. They were found to occur on the skin surfaces of approximately 25% of military recruits and healthy males. Al-Khoja and Darrell used contact plates to investigate the skin flora of the antecubital fossa of hospital inpatients and healthy people and found *Acinetobacter* spp. in 23% of patients and 20% of controls (1). Those investigators speculated that these organisms frequently represent contamination if isolated from blood cultures. Gaughan et al. (12) observed that *Acinetobacter* spp. were isolated from 30% of patients with skin diseases such as eczema or psoriasis and were more frequently recovered from involved skin than from normal skin.

By using a sterile-bag sampling technique, Larson (20) found *Acinetobacter* spp. to be among the most frequently isolated gram-negative organisms recovered from the hands of hospital personnel and healthy controls. Interestingly, colonization rates of controls were nearly four times as high as those of hospital staff. *Acinetobacter* spp. have also been found occasionally (7% of subjects) in the oral cavities and respiratory tracts of healthy adults (23). Unfortunately, all of these studies were based on the old taxonomy of the genus and no information can be obtained from these reports regarding the natural reservoirs of the individual species now recognized as constituting the genus *Acinetobacter*.

To further elucidate the natural habitats of the various *Acinetobacter* genomic species, we studied skin and mucous membrane colonization of hospitalized patients and healthy volunteers and used the phenotypic properties proposed by Bouvet and Grimont (6) for identification to the species level. *Acinetobacter* strains were isolated from 75% of the patients and 43% of the controls. This rate is higher than reported in the literature, probably due to the greater number of body sites investigated here. However, since patients were investigated only once during the study, no overall conclusion can be drawn as to the rate of persistent versus transient skin carriage. Persistent carriage of *Acinetobacter* spp. has been observed previously in hospital inpatients by Dijkshoorn et al. (10).

TABLE 4. Comparison of phenotypic and genotypic methods for the identification of *Acinetobacter* spp.

Strain no.	DNA-DNA hybridization	Phenotypic identification	ARDRA[a]	SDS-PAGE	*Eco*RI ribotype
SH 046	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 047II	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 063	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 078	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 084	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 088	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 093	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 101II	DNA group 8	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*
SH 106	DNA group 8	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*
SH 112	DNA group 8	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*
SH 134	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 139	Not DNA groups 1–15TU	Not identified	Not identified	Not identified	Not identified
SH 145	DNA group 8	*A. lwoffii*	*A. lwoffii*	DNA group 7/8	*A. lwoffii*
SH 147	DNA group 8	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*
SH 148	DNA group 15TU	Biochemically inactive	DNA group 15TU	*A. johnsonii*	DNA group 15TU
SH 180	DNA group 7	*A. johnsonii*	Not identified	*A. johnsonii*	*A. johnsonii*
SH 184	DNA group 8	Biochemically inactive	*A. lwoffii*	*A. lwoffii*	*A. lwoffii*
SH 196	DNA group 7	*A. johnsonii*	Not identified	*A. johnsonii*	*A. johnsonii*
SH 203	Not DNA groups 1–15TU	*A. lwoffii*	Not identified	Not identified	Not identified
SH 206	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 209	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 213	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 214	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*
SH 219	Not tested	Atypical	DNA group 3	DNA group 3	DNA group 3
SH 232	DNA group 7	*A. johnsonii*	"*A. johnsonii*"	*A. johnsonii*	*A. johnsonii*

[a] "*A. johnsonii*" signifies an "atypical" ARDRA pattern; presumptive identification.

Interestingly, the number of colonized body sites was four times as great in patients as in controls. We speculate that this may be due to the warm and humid atmosphere in the patients' beds and to the fact that most patients probably shower and bathe less frequently than healthy controls. This assumption is also supported by the fact that patients investigated during their second week in the hospital were colonized more frequently than patients studied during their first days of hospitalization (data not shown). However, our observations may be biased by the different age and sex distributions in patients and controls; thus, no final conclusions can be drawn. Taplin et al. have also suggested a high moisture requirement for survival and multiplication of *Acinetobacter* spp. (30). This requirement was thought to be at least one of the reasons why acinetobacters were more prevalent on skin during the humid summer months in the study of Kloos and Musselwhite (19), as well as for the increased frequency of nosocomial infections due to *Acinetobacter* spp. in the hot season observed by Retailliau et al. (22). No seasonal variation was observed in this survey, but the climate in Germany is not sufficiently constant to make a comparison of "summer" and "winter" samples meaningful.

Acinetobacter spp. occurred mostly in only small numbers on human skin, based on the number of CFUs obtained on primary isolation plates. The estimated bacterial density was usually 2 $\log_2$ less than that of gram-positive skin organisms such as coagulase-negative staphylococci. This compares with the observation of Kloos and Musselwhite, who found that *Acinetobacter* spp. comprised less than 1% of the total bacteria isolated from nares and axillae (19). Colonization rates for specific body sites ranged from 15 to 40% in patients and from 0 to 20% in controls, with no significant differences by site except that the throat was found to be colonized only rarely in patients and not at all in controls.

The species recovered most commonly from the skin of both inpatients and controls were *A. lwoffii*, *A. johnsonii*, and *Acinetobacter* DNA group 3. *A. radioresistens*, in contrast, was recovered frequently from patients but not at all from controls. We have no explanation for this difference. Al-Khoja and Darrell (1) found *A. calcoaceticus* subsp. *lwoffii* nearly three times more frequently than *A. calcoaceticus* subsp. *anitratus* on human skin. Since multiple *Acinetobacter* genomic species are glucose nonacidifying, it is not certain whether all isolates were truly *A. lwoffii* according to current taxonomy. *A. lwoffii* and *A. johnsonii* have also been isolated from animals, as well as from food sources (13), whereas *A. johnsonii* was also found in soil and activated sludge (2, 11). *Acinetobacter* DNA group 3 has been found in both clinical and nonclinical samples (2, 26, 29, 32). Apart from catheter-related infections, all of these species are implicated only rarely in clinical infections (25, 26). *A. baumannii* and DNA group 13TU are the species associated most commonly with nosocomial infection (73 to 90% of *Acinetobacter* isolates from patients; 5, 26). However, these species were documented only once and twice on human skin, respectively. This observation negates the common opinion that nosocomial infections due to *Acinetobacter* spp. are frequent simply because acinetobacters are ubiquitous in the environment and are natural inhabitants of human skin. It is of note that one of the two DNA group 13TU isolates was recovered from a technician working with *Acinetobacter* and was confirmed by pulsed-field gel electrophoresis to represent a known outbreak strain (data not shown). It therefore appears that *A. baumannii* is probably introduced only rarely de novo from the community into the hospital setting by a newly admitted patient, and its natural source still remains to be defined.

Cross transmission of *Acinetobacter* spp. other than *A. baumannii* in hospitals outside of the intensive care setting is probably rare. No endemic strain was detected during the study period, and the same strain (*A. lwoffii*) in two patients, as determined by plasmid analysis, was documented only once. However, to adequately assess the extent of cross transmission, a prospective study would be required with much more frequent sampling of all patients admitted to the cardiology ward.

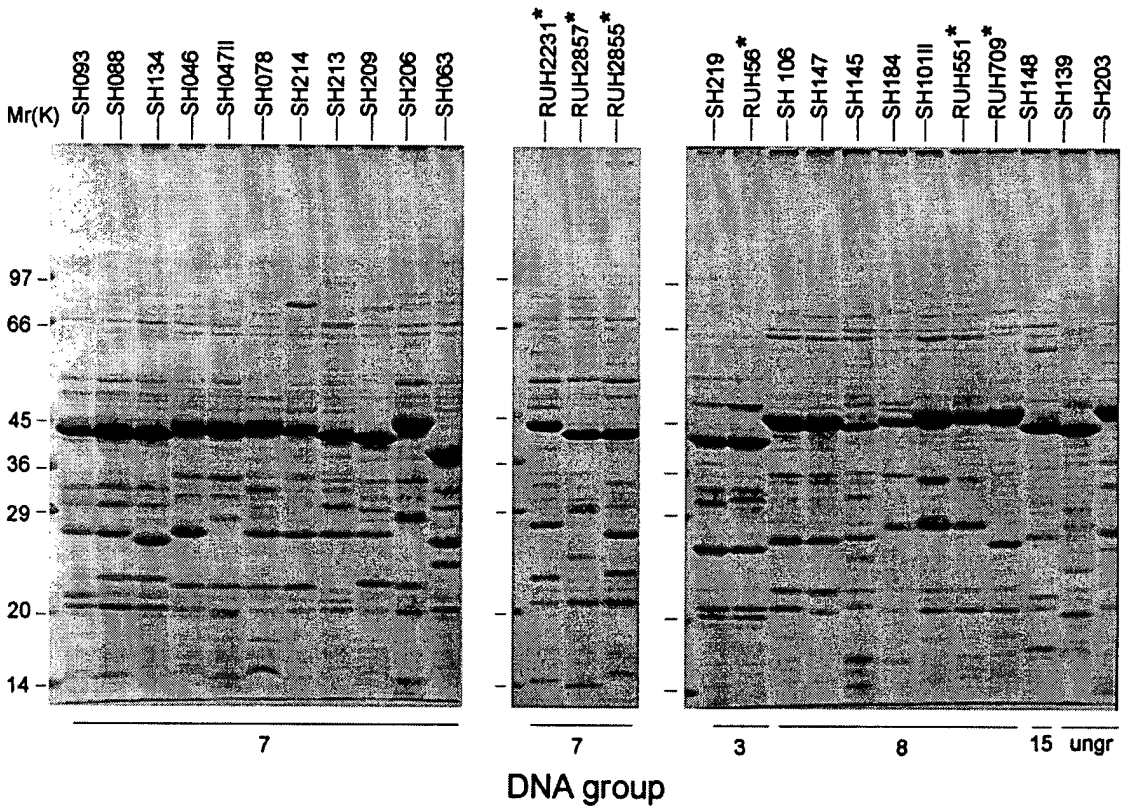

FIG. 3. Cell envelope protein profiles of 20 *Acinetobacter* isolates of the present study (Table 4). *, profiles of RUH reference strains used for presumptive DNA group identification; ung, ungroupable. K, in thousands.

ARDRA is a relatively simple method for genomic species identification which is based on the detection of differences in 16S ribosomal DNA without the need for DNA sequencing. The method was recently described for *Acinetobacter* (33), and a comprehensive database of strains previously allocated to DNA groups by DNA-DNA hybridization is being built up.

Apart from the problems with *A. johnsonii*, all identifications by ARDRA were correct. By addition of new combinations of patterns (i.e., *Cfo*I 5 or *Cfo*I 1 and 5 in combination with *Alu*I 4 and *Bfa*I 1) observed in this study for *A. johnsonii*, similar isolates can be recognized in the future. The phenomenon of mixed patterns was recently observed in other isolates of another collection presently under study (10a). An explanation may be that multiple copies of the 16S ribosomal gene with minor variations in nucleotide sequences resulting in the mixed ARDRA patterns occur in these strains. Only two isolates, i.e., those with an aberrant *Bfa*I pattern, could not be identified as *A. johnsonii* by ARDRA since differentiation with *A. haemolyticus* and DNA group 14TU was impossible.

Biochemical identification performed well, and only three isolates were misidentified (two DNA group 13TU isolates were misidentified as *A. baumannii*, and one ungroupable isolate was identified as *A. lwoffii*), while only three isolates (one in DNA group 3, one of *A. lwoffii*, and one in DNA group 14TU) which were biochemically atypical or inactive were not identified. It is well known that *A. baumannii* and DNA group 13TU cannot be differentiated phenotypically (15). Only two isolates (SH 203 and SH 139) could not be assigned to any of the known DNA groups by any of the methods used. This indicates that current taxonomic knowledge covers most of the species that occur commensally and also agrees with a previous study showing that biochemical identification was most successful for *A. baumannii*, *A. johnsonii*, *A. lwoffii*, and *A. radioresistens*, with more than 90% of the strains identified (15).

For all isolates of *A. junii*, *Acinetobacter* DNA group 10, and *A. radioresistens*, identifications by both techniques were in agreement. From this, it can be concluded that overall agreement between phenotypic identification and ARDRA was good and that both methods performed equally well.

Comparison of the results obtained with various identification methods, i.e., DNA-DNA hybridization, phenotyping, ARDRA, SDS-PAGE, and ribotyping for a selected number of isolates showed good agreement for most strains.

Identification based on cell envelope protein SDS-PAGE was performed without consideration of the isolate identification results obtained by other methods. The patterns obtained previously for a collection of 120 strains of DNA groups 1 to 12, 13TU, and 14TU (9) were used as a library for visual comparison and identification to the genomic species level by two individuals. DNA groups 13 to 17BJ and 15TU were not included in the reference collection, which may explain the wrong identification of SH 148 (which belonged to DNA group 15TU). Apart from strain SH 145 (identified as belonging to DNA group 7/8), identification of the other isolates was in agreement with DNA hybridization results. Numerous studies have shown that protein electrophoretic patterns are useful taxonomic markers (21, 34), and the results of the present study show that this is also the case for *Acinetobacter* spp. However, the heterogeneity of patterns within the genus *Acinetobacter* is considerable and allocation of strains to particular genomic species by visual analysis is a subjective process. Improvement may be obtained by computer-assisted analysis of

digitized patterns with commercially available software packages, as in a previous taxonomic study (9). Therefore, it can be concluded that *Acinetobacter* genomic species identification based on protein electrophoretic patterns, although in good agreement with DNA hybridization, is not the method of choice in most laboratories.

The present study is the first to apply ribotyping to identification of *Acinetobacter* spp. outside the *A. calcoaceticus-A. baumannii* complex. The results showed good agreement with other genotypic identification methods. The patterns were quite complex, although bands specific to particular DNA groups were seen. However, if the method is to be used only for identification (and not for discrimination below the DNA group level), this technique is too complicated to be really useful if simpler methods are available.

In conclusion, human skin appears to be a natural habitat of certain *Acinetobacter* species, in particular, *A. lwoffii*, *A. johnsonii*, *Acinetobacter* DNA group 3, and probably *A. radioresistens*, although the latter species was found only in hospitalized patients. It is therefore not surprising that these species in particular have been recovered from blood cultures of patients with catheter-related bloodstream infections (27). *A. baumannii* and *Acinetobacter* DNA group 13TU, the most important nosocomial *Acinetobacter* spp. were, in contrast, found only rarely on human skin. Both the frequency of colonization and the number of body sites colonized with *Acinetobacter* species were greater in patients than in controls. The extent of colonization with *Acinetobacter* spp. seems to increase during the course of hospitalization. The phenotypic identification scheme of Bouvet and Grimont allowed correct identification at the species level in most instances and showed good agreement with ARDRA and with other, more laborious techniques. Unfortunately, none of these techniques is suitable for routine use.

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Studies on bioemulsifier production by *Acinetobacter* strains isolated from healthy human skin

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Aims: In recent years, interest has been growing in the search for novel bioemulsifiers. Many bacterial genera including *Acinetobacter* have been reported to produce bioemulsifiers. The present study aims to screen *Acinetobacter* isolates from healthy human skin for bioemulsifier production.

Methods and Results: *Acinetobacter junii* SC14 produced maximum bioemulsifier in the presence of almond oil during stationary growth phase at 37°C and pH 7·2. Partially purified, nondialysable bioemulsifier from SC14 was a proteoglycan. The protein and polysaccharide fractions resulted in 95·2% reconstitution of the emulsification activity. The role of esterase in the release of cell-bound emulsifier and the contribution of capsular polysaccharide to the emulsification activity were observed.

Conclusion: *Acinetobacter* strains from human skin exhibited better emulsification activity than that by burn wound or soil isolates, owing to the inherent differences in chemical microenvironment of their habitats.

Significance and Impact of the Study: Investigation of skin commensals, especially acinetobacters, would lead to the discovery of novel bioemulsifiers with interesting properties. Attempts of screening and strain improvement directed towards skin commensals will open up new avenues for strains producing bioemulsifier on a commercial scale.

INTRODUCTION

Growth of micro-organisms on water-insoluble carbon sources is often accompanied by emulsification of the water-insoluble substrate in the culture medium by virtue of the production of bioemulsifiers (Zajic and Panchal 1976; Rosenberg 1986). Many amphipathic molecules produced by living cells possess surface-active characteristics observed in the form of lowering of interfacial tension at the oil–water interface which leads to the formation of microemulsions (Gutnick and Minas 1987). Bioemulsifiers are an example of such surface-active agents of microbial origin. Bioemulsifiers are amphipathic molecules playing a vital role in microbial growth on hydrophobic substrates. These bioemulsifiers find potential applications in cosmetic, food, agricultural and pharmaceutical industries (Rosenberg 1986). Moreover, health hazards of oil spills can also be minimized through bioremediation employing specific microorganisms (Gutnick and Rosenberg 1977).

Among a variety of bacterial genera studied, production of extracellular emulsifiers has been observed to be a phenomenon of common occurrence among the members of the genus *Acinetobacter* (Rosenberg 1986). Emulsan, the extracellular polyanionic bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1 has been studied in great detail (Rosenberg *et al.* 1979a, b; Zuckerberg *et al.* 1979; Rubinovitz *et al.* 1982; Zosim *et al.* 1982; Pines and Gutnick 1986; Foght *et al.* 1989). Many strains of *Acinetobacter* isolated from soil, mud, marine water, fresh water, etc. have been reported to produce bioemulsifiers (Sar and Rosenberg 1983; Foght *et al.* 1989). Moreover, a few reference strains, viz. *Ac. calcoaceticus* BD4 and BD413, ATCC 17294, ATCC 17906, were also investigated for emulsifier production (Kaplan and Rosenberg 1982; Kaplan *et al.* 1987). However, there is no report of bioemulsifier production by *Acinetobacter* strains from human skin. Acinetobacters can be isolated from the skin

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of around 40% of the human population with recovery of 7–18 genospecies but the dominant isolates include *Ac. lwoffii*, *Ac. junii*, *Ac. johnsonii* and *Ac. calcoaceticus* (Seifert *et al.* 1997; Berlau *et al.* 1999; Chu *et al.* 1999). The presence of numerous hydrophobic substances on human skin in the form of skin surface lipids (SSL) (Strauss *et al.* 1975; Downing *et al.* 1999) necessitates that the skin microflora be able to resist the inhibitory action of SSLs and, if possible, be capable of metabolizing these substrates. In view of this background, bioemulsifier production by *Acinetobacter* strains isolated from healthy human skin was studied. Apart from these naturally occurring moieties, a considerable fraction of oils can be contributed to the skin microenvironment in the form of cosmetics. One can find substantial documentation of the use of oils, viz. almond oil, castor oil, olive oil, palm oil, etc., in the manufacture of cosmetics, especially the skin-care products (Hoshizaki and Suzuki 1978; De Vries 1987; Horie 1988; Szepessy *et al.* 1990). These facts prompted us to screen skin *Acinetobacter* strains for bioemulsifier production. The present paper describes bioemulsifier production by *Acinetobacter* strains from healthy human skin, partial purification and characterization of the bioemulsifier, and a study of the effect of physicochemical factors on its production and activity.

MATERIALS AND METHODS

Source and biotyping of bacterial strains used

Twelve *Acinetobacter* strains consisting of eight from healthy human skin, and two each from burn wound and soil were used in the present study. The burn wound and soil isolates were used for comparison purposes. These isolates were obtained after enrichment of respective samples in Baumann's enrichment medium (Baumann 1968). Identification of the isolates up to genus level was carried out as per the chromosomal DNA transformation assay (Juni 1972). Delineation of the isolates into various genospecies was performed as per the biochemical scheme recommended by Bouvet and Grimont (1986, 1987). The control strains used included *Acinetobacter calcoaceticus* ATCC 33305, *Ac. calcoaceticus* EBF 65/65 C426, *Ac. calcoaceticus* EBF 65/65 C4169, *Ac. calcoaceticus* BD4, *Ac. calcoaceticus* BD413, *Ac. calcoaceticus* MTCC 127, *Ac. calcoaceticus* MTCC 1271, *Ac. calcoaceticus* MTCC 1425, *Ac. lwoffii* MTCC 496, *Escherichia coli* MTCC 68, *E. coli* K12 HB101, *E. coli* K12 DH5α, *E. coli* K12 JM103, *Pseudomonas fluorescens* MTCC 740, *Ps. putida* MTCC 1313 and *Staphylococcus aureus* MTCC 740.

Oils used as substrates

The following oils were used as substrates during fermentation: almond oil from Hamdard (WAKF) Laboratories, Ghaziabad, UP, India; castor oil from Shree Krishna Pharmacy, Mumbai, India; olive oil from Campbell Agro Mfg Industries Pvt. Ltd, Mumbai, India; and palm oil from Chakan Oil Mills, Chakan, India. All oils were of analytical grade.

Bioemulsifier production

The mineral salt medium (BNP) used (Foght *et al.* 1989) consisted of (l^{-1} solution) 0·5 g K_2HPO_4, 1 g NH_4Cl, 2 g Na_2SO_4, 2 g KNO_3, 0·2 g $MgSO_4.7H_2O$, and 0·002 g $FeSO_4.7H_2O$. The medium was supplemented with 1 g l^{-1} peptone. The pH of the medium was adjusted to 7·2. Bioemulsifier production was carried out in 50 ml of above medium in a 250-ml Erlenmeyer flask at 37°C with shaking at 150 rev min^{-1}. Soil isolates were grown at 30°C. Bacterial growth was initiated by the introduction of 1% (v/v) inoculum growing exponentially in the same medium. The desired quantity of respective test oil (v/v) was added to the cooled fermentation medium after autoclaving. Samples withdrawn at 5 h interval were first used to record the culture density at 660 nm and then used for the emulsification assay.

Emulsification assay

Three ml of cell-free culture broth or a suitably diluted emulsifier preparation were mixed with 0·5 ml test oil, vortexed vigorously for 2 min and incubated at 37°C for 1 h. Absorbance of the aqueous phase was then recorded at 400 nm. The absorbance maxima was arrived at after scanning the entire visible light spectrum (UV-1601 Shimadzu Corporation, Japan). The blank was prepared similarly with sterile production medium replacing the cell-free culture broth or the emulsifier aliquot. An absorbance of 0·010 units at 400 nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU ml^{-1}).

Stability of emulsion

After 1 h incubation of the vortexed mixture of test oil and emulsifier at 37°C, the absorbance of aqueous layer at 400 nm was recorded. One set was incubated at 37°C and the other at 10°C. Absorbance of aqueous layer was noted every 20 h (Zosim *et al.* 1982).

Effect of physico-chemical factors on bioemulsifer production and activity

Effect of temperature (20, 30, 40 and 50°C) on bioemulsifier production was studied by growing the test strain at the respective temperature in a shaking water bath at

150 rev min^{-1} and then checking the culture supernatant for oil emulsification as per the method described above. Effect of temperature on bioemulsifier activity was studied by incubating the vortexed cell supernatant and oil mixture at the respective temperatures for 1 h and then recording the absorbance of the aqueous layer at 400 nm.

Effect of pH (4, 5, 6, 7, 8 and 9) on bioemulsifier production and activity was studied similarly. The optimum temperature of 37°C was used in all experiments hereafter unless stated otherwise. The fermentation medium was supplemented with required quantities of one of the three test salts (NaCl, $CaCl_2$, $MgCl_2$) to study the effect of salts on bioemulsifier production. Effect of salts on bioemulsifier activity was tested by incubating the bioemulsifier sample in presence of required quantity of test salt at 37°C for 1 h and then using it in the standard emulsification assay.

Effect of inducer oil on bioemulsifier production

Growth of *Acinetobacter* strains was allowed in BNP medium supplemented with 1% of test oils separately. The resulting cell-free supernatants were assayed for emulsification of each test oil according to the emulsification assay described above.

Partial purification of bioemulsifier

Acinetobacter junii SC14 (henceforth referred to as SC14) producing maximum levels of bioemulsifier was selected for further study. The cell-free culture broth obtained by centrifugation at 8000 rev min^{-1} for 20 min was mixed with three volumes of chilled acetone and incubated at 4°C for 15 h. The brown precipitate was collected by centrifugation at 10 000 rev min^{-1} for 30 min and dissolved in minimum volume of sterile distilled water (pH 7). This solution was then dialysed (Seamless cellulose tubing, width 40 mm, diameter 25 mm, retaining most proteins of molecular weight 12 000 or greater, Sigma Aldrich Chemie, Gmbh, Steinheim, Germany) extensively against sterile distilled water at 10°C for 48 h. The distilled water in dialysis container was replaced every 10 h. The dialysate was then frozen at −20°C and lyophilized. This bioemulsifier preparation was stored in airtight glass vials at room temperature (30°C).

Chemical analysis of bioemulsifier

A portion of the partially purified bioemulsifier was used for chemical analysis. Protein content was assayed using the method described by Lowry *et al.* (1951) with bovine serum albumin as standard. Carbohydrates were quantified according to the protocol proposed by Dubois *et al.* (1956). Reducing sugar content was estimated using the dinitro salicylic acid method (Monreal and Reese 1969) with glucose as a standard. Extraction and quantification of lipids was performed as per the method described by Reddy *et al.* (1983).

Determination of viscosity

Different aliquots (0·25–3·0 ml) of solution of the partially purified bioemulsifier (5 mg ml^{-1}) were used to emulsify a fixed volume of almond oil (6 ml). The viscosity of these emulsified oil samples (6 ml) was recorded using a standard viscometer at 25°C. Readings were taken in duplicate and the average was reported. Unemulsified almond oil was used as the control.

Determination of esterase activity

Quantification of the esterase activity was performed according to the colorimetric assay protocol described by Shabtai and Gutnick (1985) with *p*-nitrophenyl acetate as the substrate. Cell pellet as well as the cell-free supernatant was assayed for esterase activity after every 10 h during the 60 h long fermentation.

Reconstitution of emulsification activity

On the basis of chemical composition of the partially purified emulsifier, the components were isolated. The protein and polysaccharide fractions from the bioemulsifier (50 mg) and the cell-free supernatant (300 ml) were isolated. Protein fraction of the purified bioemulsifier was prepared by hot phenol treatment. Extracellular protein was obtained by 60% ammonium sulphate precipitation of the cell-free culture broth. The polysaccharide fraction from purified bioemulsifier was isolated by water extraction of phenol phase, while the capsular polysaccharide was obtained by acetone precipitation of the homogenized culture supernatant (Kaplan *et al.* 1987). These fractions were then checked for their ability to reconstitute the almond oil emulsification property, individually or in combination, according to the emulsification assay described above.

Cell-surface hydrophobicity assay

Cell-surface hydrophobicity of the test strains was determined using hexadecane and almond oil (Rosenberg *et al.* 1980) with slight modification. The volume of test hydrocarbon used ranged from 0·1 to 1·6 ml. Incubation of the culture and hydrocarbon was carried out at 37°C instead of 30°C, except in the case of the soil isolate. *Acinetobacter baumannii* SB-1 isolated from burn wound (referred to as SB-1) and *Ac. calcoaceticus* GS1LB isolated from soil (referred to as GS1LB) were also tested. *Escherichia coli*

MTCC 68 (referred to as EC68) and *Pseudomonas aeruginosa* MTCC 1223 (referred to as PA1223) were used as controls.

RESULTS

Biotyping of *Acinetobacter* strains

Out of eight skin isolates selected, two were identified to be *Ac. baumannii*, two as *Ac. lwoffii*, two as *Ac. junii* and the remaining two as *Ac. haemolyticus*. The burn wound isolates were observed to belong to *Ac. baumannii*, while the soil isolates were observed to be *Ac. calcoaceticus*. The chromosomal DNA transformation assay was useful in confirming the generic identity of the isolates, while the biochemical scheme proposed by Bouvet and Grimont (1986, 1987) helped us in delineation of the isolates into various *Acinetobacter* genospecies.

Bioemulsifier production by acinetobacters from human skin

Among the eight *Acinetobacter* strains isolated from human skin tested for bioemulsifier production, *Ac. junii* SC14 isolated from healthy human skin exhibited maximum bioemulsifier production, followed by *Ac. baumannii* (Fig. 1). *Acinetobacter haemolyticus* emulsified olive oil to the minimum extent, while palm oil was minimally emulsified by *Ac. junii*. In general, almond oil was emulsified to the maximum extent, while palm oil was least emulsified. SC14 emulsified almond oil to the maximum extent. The kinetics of bioemulsifier production and growth pattern of SC14 is shown in Fig. 2. Fermentation for 30 h under given set of conditions led to production of 116·6 EU ml^{-1} in presence of 1% almond oil.

Stability of emulsion

The almond oil-in-water emulsion was observed to be highly stable. The emulsion maintained 90% of its stability up to 6 d at 37°C, after which phase separation was observed. However, 30% loss of stability was noted within 20 h at 10°C. The emulsion retained only 35% stability after 140 h at 10°C.

Effect of physico-chemical factors

In the case of SC14, fermentation at 37°C and pH 7·2 in presence of 0·5% NaCl was observed to yield the highest levels of bioemulsifier (Fig. 3a). It was found that at 50°C, more than 50% bioemulsifier activity was inhibited, making it comparatively less thermostable. Incubation at 37°C and pH 7·2 also were optimum for the emulsification activity. However, 1% concentration of NaCl, $CaCl_2$ and $MgCl_2$, used separately, caused 31·3, 43·6 and 17·3% inhibition of the emulsification activity, respectively (Fig. 3b).

Substrate specificity of bioemulsifier

Among the four oils tested, almond oil served as the best substrate for emulsification for all the strains tested (Fig. 1). Palm oil was emulsified to the least extent. Also, maximum emulsification of almond oil was observed irrespective of the oil used during fermentation (Table 1), with the exception of castor oil. Strain SC14 was observed to tolerate

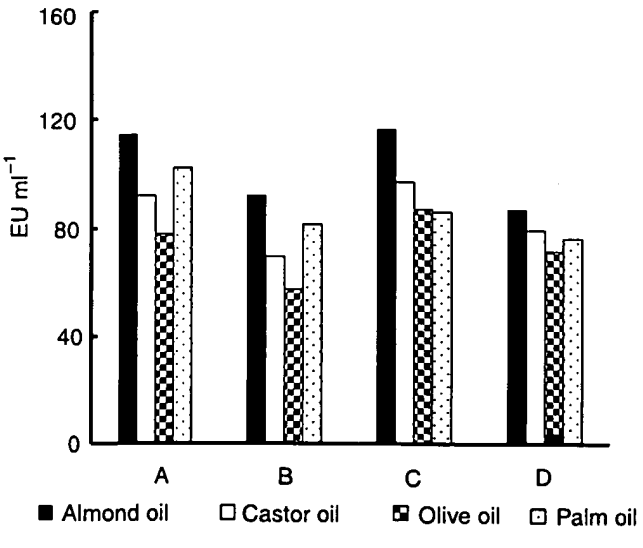

Fig. 1 Emulsification of test oils by different *Acinetobacter* strains representing four different genospecies. A: *Acinetobacter baumannii*; B: *Acinetobacter haemolyticus*; C: *Acinetobacter junii*; D: *Acinetobacter lwoffii*

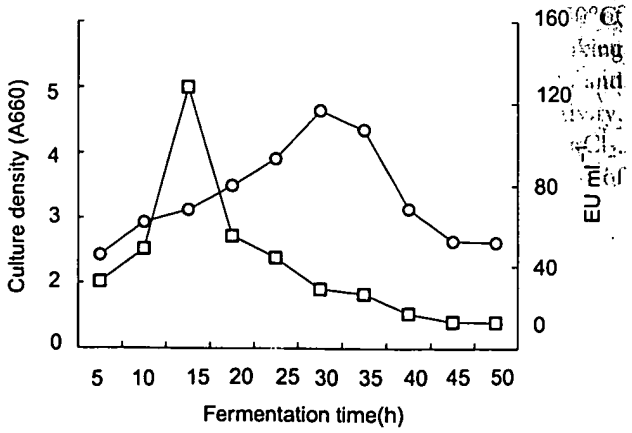

Fig. 2 Time course of cell growth and bioemulsifier production by *Acinetobacter junii* SC14 at 37°C in the presence of 1% almond oil. □, A660; ○, EU ml^{-1}

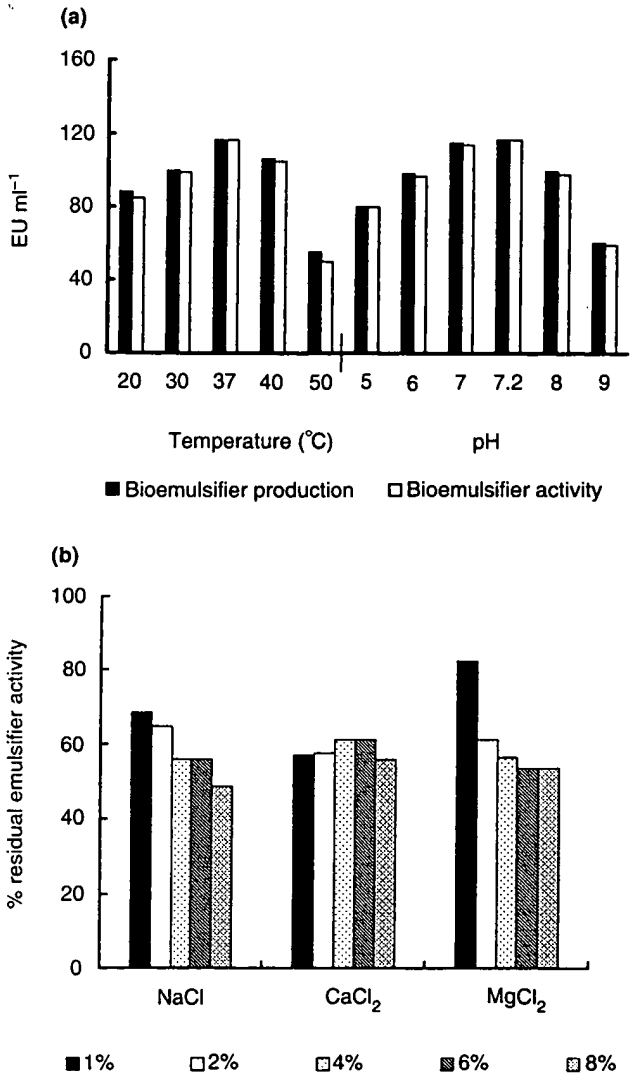

Fig. 3 (a) Effect of temperature and pH on bioemulsifier production and activity by *Acinetobacter junii* SC14. (b) Effect of salts on activity of bioemulsifier produced by *Acinetobacter junii* SC14

Table 1 Effect of inducer oil on bioemulsifier production by *Acinetobacter junii* SC14

Growth in 1% of*	Emulsification of (U ml^{-1})			
	Almond oil	Castor oil	Olive oil	Palm oil
Almond oil	116·6	72·0	66·4	85·0
Castor oil	66·1	97·1	69·1	79·0
Olive oil	99·5	85·6	87·1	85·1
Palm oil	90·3	79·1	80·1	86·1

*Fermentation medium supplemented with 1% test oil.

up to 40% almond oil, but the optimum bioemulsifier production took place in the presence of 18% almond oil (427·8 EU ml^{-1}).

Partial purification of bioemulsifier

The partially purified bioemulsifier obtained from cell-free supernatant of SC14 culture grown in presence of 18% almond oil exhibited 1759·8 EU ml^{-1}. The bioemulsifier yield was 3·9 g l^{-1} under given set of conditions. Chemical analysis of this bioemulsifier revealed that protein (50·5%) was the major constituent, followed by polysaccharide (43%). It was noted that 88·7% of the polysaccharide comprised of reducing sugars. A minor fraction of lipid (3·8%) was also detected in the bioemulsifier. Thus proteoglycan nature of the bioemulsifier produced by SC14 is evident from its chemical composition.

Esterase activity

The kinetics of esterase production by SC14 is shown in Fig. 4. The highest esterase activity (122·8 U mg^{-1} of protein) was found to be cell-associated at 30 h, at which time the extracellular broth also showed esterase activity (77·4 U ml^{-1}) during the 60 h fermentation. Notably 61·3% of the peak esterase activity was observed to be cell-associated, while only 38·6% activity was secreted into the fermentation medium. Moreover, it was observed that throughout the fermentation period, significant esterase activity was observed to be associated with the cells while only a minor fraction was detected in the fermentation broth.

Viscometry

Increase in bioemulsifier concentration from 0·5 ml to 3 ml against a fixed volume of 6 ml almond oil resulted in a reduction in viscosity of almond oil by 40·3%.

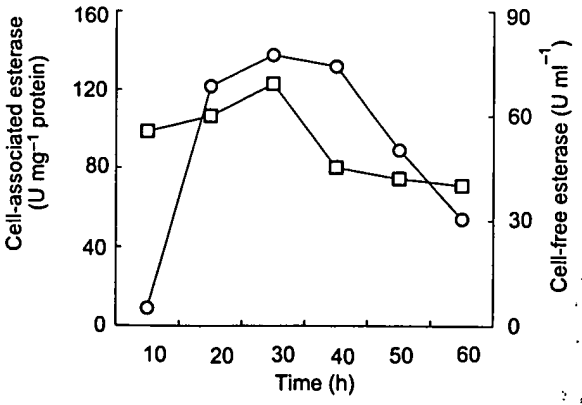

Fig. 4 Esterase production by *Acinetobacter junii* SC14. □, cell pellet; ○, cell-free supernatant

Cell-surface hydrophobicity

Degree of adherence of SC14, SB-1, 1LB, EC78 and PA1223 to almond oil and hexadecane is shown in Fig. 5a,b. *Acinteobacter junii* SC14 showed lower affinity towards almond oil as well as hexadecane as compared to its counterparts from burn wound and soil. Even the control strains of *E. coli* and *Ps. aeruginosa* had higher affinity for almond oil and hexadecane than SC14. Thus the cell surface of SC14 appeared to be less hydrophobic than that of the other test and control strains.

Reconstitution of bioemulsifier activity

Table 2 summarizes the percentage emulsification activity reconstituted in the presence of the given fractions using almond oil as the substrate. The fractions did show emulsification activity independently, but the activity was enhanced when all the fractions were present together. Polysaccharide fractions from the bioemulsifier and extracellular broth showed lower emulsifying activity than the extracellular protein and the bioemulsifier protein. It should be noted that a prominent minicapsule was evident during the logarithmic growth phase of SC14, while only traces of the capsule were seen on the cell surface during the stationary phase.

Table 2 Reconstitution of emulsification activity by isolated fractions of the bioemulsifier produced by *Acinetobacter junii* SC14

Fraction	% activity	EU ml^{-1}
Purified emulsifier	100	1759·8
BEP*	78·4	1380·0
EP†	78·2	1377·2
BEPS‡	74·5	1312·2
EPS§	76·2	1342·5
BEP + BEPS	84·7	1473·9
BEP + EP	78·0	1372·7
BEP + EPS	81·0	1425·0
BEPS + EPS	74·4	1310·6
BEP + BEPS + EP + EPS	95·2	1676·2

BEP: bioemulsifier protein; EP: extracellular protein; BEPS: bioemulsifier polysaccharide; EPS: exopolysaccharide.

*Protein fraction of the purified bioemulsifier prepared by hot phenol treatment.

†Extracellular protein obtained by 60% ammonium sulphate precipitation of cell-free culture broth.

‡Polysaccharide fraction from purified bioemulsifier isolated by water extraction of phenol phase.

§Capsular polysaccharide precipitate obtained by acetone precipitation of the homogenized culture supernatant.

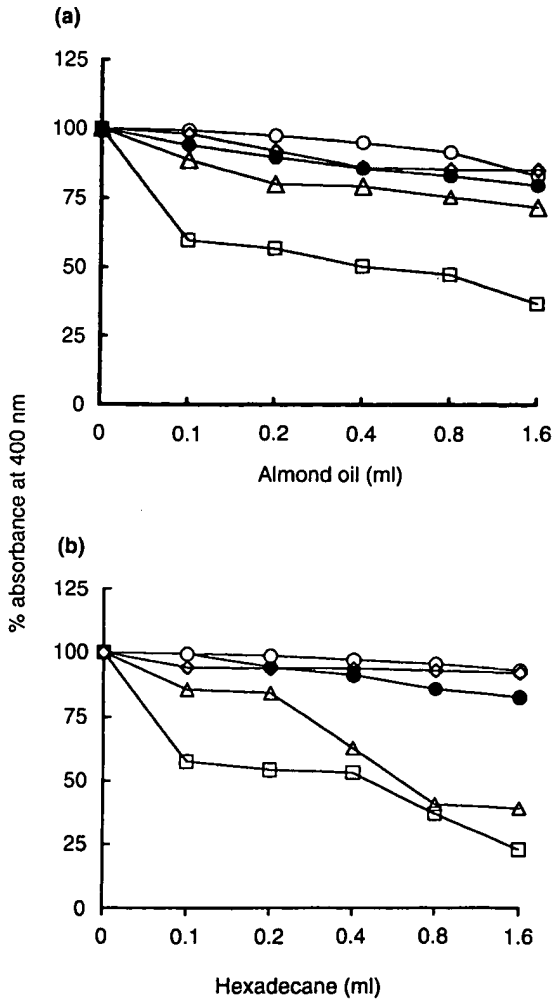

Fig. 5 Cell-surface hydrophobicity of *Acinetobacter* strains using (a) almond oil and (b) hexadecane as test substrate. ○, SC14; □, SB-1; △, GS1LB; ●, EC78; ◇, PA1223

DISCUSSION

Acinetobacter junii SC14 from human skin exhibited the maximum bioemulsifier production. Peak bioemulsifier production by SC14 was observed at 30 h at 37°C under a given set of conditions. This period coincides with the mid-stationary growth phase of the strain. Fermentation for an extended period of 30 h caused a sharp decline in the bioemulsifier production to less than 50% of the peak value. Rosenberg *et al.* (1979a) have also reported maximum emulsan production by *Ac. calcoaceticus* RAG-1 during the stationary growth phase. Bioemulsifier production by *Ac. calcoaceticus* RAG-1 can be attributed to its metabolic requirement of ability to grow on various hydrophobic substances prevalent in the marine environment. Similarly, the human skin does receive many hydrophobic compounds in the form of SSLs which are a mixture of triglycerides, fatty acids, wax esters, squalene, cholesterol and cholesterol

esters (Strauss *et al.* 1975; Downing *et al.* 1999). Human skin is also exposed to exogenous oily compounds in the form of skin-care cosmetics which contain almond oil, castor oil, olive oil and palm oil (Hoshizaki and Suzuki 1978; De Vries 1987; Horie 1988; Szepessy *et al.* 1990). In this way, the presence of various hydrophobic substances on human skin can be a predisposing factor for the emulsification ability of the resident microbes.

Chemical composition of the test oils (Budavari *et al.* 1996) was also taken into consideration during study of the bioemulsifier. All the test oils comprised mainly of three fatty acids, viz. oleic acid, linoleic acid and palmitic acid, in varying proportions. Oleic acid and linoleic acid are unsaturated fatty acids, whereas palmitic acid, stearic acid and arachidic acid are saturated fatty acids. Accordingly, almond oil and castor oil display a higher degree of unsaturation as compared to olive and palm oil. Still, almond oil was attacked most readily by the test strains, whereas palm oil, with the least degree of unsaturation, was not easily emulsified.

Optimum temperature for bioemulsifier production was found to be 37°C, which is also suggestive of its potential role in growth of skin bacteria. More than 50% loss of activity of the present bioemulsifier at 50°C is contrary to the observation of heat activation of alasan (Navon-Venezia *et al.* 1995). Emulsan is reported to be maximally produced and active at 30°C since the producer strain, *Ac. calcoaceticus* RAG-1, is an environmental isolate (Rosenberg *et al.* 1979a). Slightly alkaline pH of 7·2 was found to be optimum for bioemulsifier production, while a slightly acidic pH of 6·5 allowed maximum bioemulsifier activity. Human skin is generally acidic (pH 5·6–5·8), but the present bioemulsifier showed a near neutral pH optimum. This variation can be due to the physicochemical differences between the *in vivo* and *in vitro* systems. In the case of alasan produced by *Ac. radioresistens* KA53 (Navon-Venezia *et al.* 1995), activity was observed over a wide pH range of 3·3–9·2, with an optimum at pH 5. The bioemulsifier investigated in the present study exhibited 80 EU ml^{-1} at pH 5. However, at pH 9, about 48% of the bioemulsifier production and activity was inhibited. It indicates that higher bioemulsifier production and activity are attainable in the acidic pH than the alkaline pH. Among the three salts tested, $CaCl_2$ caused stronger inhibition of bioemulsifier activity than NaCl and $MgCl_2$. In the presence of 2% $CaCl_2$ and $MgCl_2$, bioemulsifier production remained unaffected, while the activity was inhibited. The same percentage of NaCl resulted in 30% inhibition of bioemulsifier activity. In the case of alasan produced by *Ac. radioresistens* KA53, magnesium ions stimulated the activity below and above the pH optimum (Navon-Venezia *et al.* 1995). Overall, none of the test salts caused considerable increase in the production and or activity of the bioemulsifier by SC14.

The almond oil in water emulsion was found to retain 90% stability at 37°C till 120 h but lost 70% stability at 10°C within 40 h. High emulsion stability at room temperature is preferable as it alleviates the need of storage at low temperature for preservation and also extends the shelf life of the product. Moreover, emulsification of almond oil by the partially purified bioemulsifier resulted in considerable reduction in the oil viscosity, which is a desired property for an emulsifier.

Cell-surface hydrophobicity assay revealed that SC14 had a slightly higher affinity for hexadecane than almond oil. Accordingly, higher emulsification of hexadecane (204 EU ml^{-1}) was observed than that of almond oil (116·6 EU ml^{-1}). However, *Ps. aeruginosa* with lower affinity for hexadecane exhibited better emulsification of hexadecane (232 EU ml^{-1}) as compared to that of the almond oil (91·9 EU ml^{-1}). *Acinetobacter baumannii* SB-1 had greater affinity for almond oil as well as hexadecane than that by SC14 but showed comparatively lower emulsification (106·3 EU ml^{-1} and 116·6 EU ml^{-1} for almond oil by SB-1 and SC14, respectively; 116·2 EU ml^{-1} and 204 EU ml^{-1} for hexadecane by SB-1 and SC14, respectively). Thus SC14, despite its lower affinity to almond oil, can utilize it in a more efficient fashion than SB-1. In an earlier report *Ps. aeruginosa* PAS 279 has been reported to display a similar pattern (Rosenberg *et al.* 1980). Thus the correlation between the affinity to hydrophobic substrate and the degree of its utilization remains to be established.

The polysaccharide fraction of the proteoglycan bioemulsifier consisted of 88·7% reducing sugars. Emulsan produced by RAG-1 has been shown to be an extracellular noncovalent complex of a lipopolysaccharide and a protein (Zuckerberg *et al.* 1979). A lipopolysaccharide emulsifier by *Acinetobacter* has also been reported (Shi and Li 1989). However, alasan produced by *Ac. radioresistens* KA53 consists of an alaninie-rich heteropolysaccharide and a protein (Navon-Venezia *et al.* 1995). Yield of alasan has been reported to be 4·6 g l^{-1} while that of the SC14 has been estimated to be 3·9 g l^{-1}.

The reconstitution experiments revealed that the capsular exopolysaccharide fraction exhibited high emulsification activity alone, pointing towards its role in the overall emulsification. A minicapsule has been demonstrated in the case of SC14. In an earlier report, *Ac. calcoaceticus* BD4, a highly encapsulated strain, was shown to produce an extracellular polysaccharide responsible for emulsifying activity (Kaplan and Rosenberg 1982). The capsule of RAG-1 has been demonstrated to be a cell-bound form of the emulsifier, emulsan (Shabtai *et al.* 1985). Disappearance of the cellular capsule during stationary growth phase and its subsequent release into the fermentation medium are attributable to the simultaneous maximum production of the bioemulsifier. All the fractions, viz, protein, polysac-

charide, extracellular protein and exopolysaccharide, are essential for optimum bioemulsifier activity. Interestingly, 95·2% (1676·2 EU ml^{-1}) reconstitution was achieved. The protein fraction of the BD4 emulsan was found to play a crucial role in emulsifying activity (Kaplan *et al.* 1987). On the contrary, in the case of RAG-1 emulsan, protein was not absolutely required for emulsifying activity.

A phenomenon of bioemulsifier accumulation on the cell surface during logarithmic growth phase and its subsequent release into the medium during stationary growth phase is reported in case of emulsan (Rubinovitz *et al.* 1982; Shabtai and Gutnick 1985; Shabtai *et al.* 1985). *Acinetobacter junii* SC14 also displays a similar trend. The pattern of esterase production by SC14 is unique. At 30 h of fermentation, at which maximum bioemulsifier production occurred, 61·3% of the total peak esterase activity was found associated with the cells and only 38·6% activity was detected in the cell-free broth. The role of esterase in the release of emulsan from the cell surface of RAG-1 has been well established, wherein a decline in cell-associated esterase was recorded to lead to a subsequent rise in the cell-free esterase and in turn to an increase in emulsan production. A fraction of cell-free esterase was also demonstrated to be associated with the emulsan (Shabtai and Gutnick 1985). On the contrary, we report simultaneous increase in cell-associated and cell-free esterase activity coincident with the maximum bioemulsifier production by SC14. Negligible activity was found associated with the bioemulsifier. Kinetics and distribution of esterase produced by SC14 suggest its involvement in the release of emulsifying factor from the cell surface into the fermentation medium. This is supported by our finding that cell pellet did not show detectable emulsifying activity (data not shown).

To summarize, the present work has demonstrated good emulsifying activity by *Acinetobacter* strains isolated from healthy human skin owing to the physicochemical micro-environment of their habitat. On the basis of promising results obtained during the present study, healthy human skin appears to be a novel source of bacteria capable of bioemulsifier production. Findings of the present study would act as a foundation on which to base similar investigations in the future.

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